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Karyotype differentiation of three anopheline taxa in the Balabacensis complex of Southeast Asia (Diptera: Culicidae)

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Abstract

Cytological studies of the mitotic and meiotic karyotypes and the polytene salivary chromosomes of *Anopheles dirus, An. takasagoensis* and *An. balabacensis* (Perlis form), have revealed significant differences in the sex chromosomes. These differences are largely due to the position of the centromere and different amounts of constitutive heterochromatin and euchromatin. The chromosomal data suggest that *An. dirus* and *An. takasagoensis* are more closely related to each other than either is to *An. balabacensis* (Perlis form). The karyological differences are very useful in differentiating these taxa, particularly the Perlis form, and lend support for their species status.

Introduction

A comparison of mitotic and meiotic karyotypes and polytene chromosomes is often useful in determining the phylogenetic affinities of closely related dipteran species, and may suggest the direction and mechanisms of chromosomal evolution (White, 1973). Accordingly, we are currently engaged in cytotaxonomic studies on members of the Leucosphyrus group of *Anopheles* in Southeast Asia. This is an assemblage in which at least three species are considered primary vectors of human malaria parasites. The present report, resulting from investigations being conducted in our laboratories on speciation within the Balabacensis complex, demonstrates that karyotype differences are also useful in the differentiation of certain taxa within the complex.

Anopheles balabacensis balabacensis Baisas has been considered one of the most important vectors of human malaria parasites in the Southeast Asian Subregion. Colless (1956, 1957) and subsequently Reid (1968) recognized at least 5 additional morphological forms or subspecies of *balabacensis* from different geographical areas of this subregion. Recently, however, evidence from morphological, crossing and cytogenetic studies have revealed that at least 4 taxa are involved in the subspecies, *b. balabacensis.* These are: *An. balabacensis,* from East Malaysia and several islands in the Philippines; *An. dirus* described from Thailand (Peyton & Harrison, 1979), which also occurs in other mainland Southeast Asian countries; *An. takasagoensis* Morishita from Taiwan, recently elevated to species status by Peyton & Harrison (1980); and the Perlis form from northern peninsular Malaysia. The present study involves only the last 3 taxa.

Material and methods

The three colonies used in this study are maintained at the Department of Medical Entomology, AFRIMS, Bangkok, and consist of: (1) *An. dirus* (Bangkok strain), colonized by Esah & Scanlon (1966) from Khao Mai Khaeo, Chon Buri Province, Thailand, but later (1971) altered by the addition of

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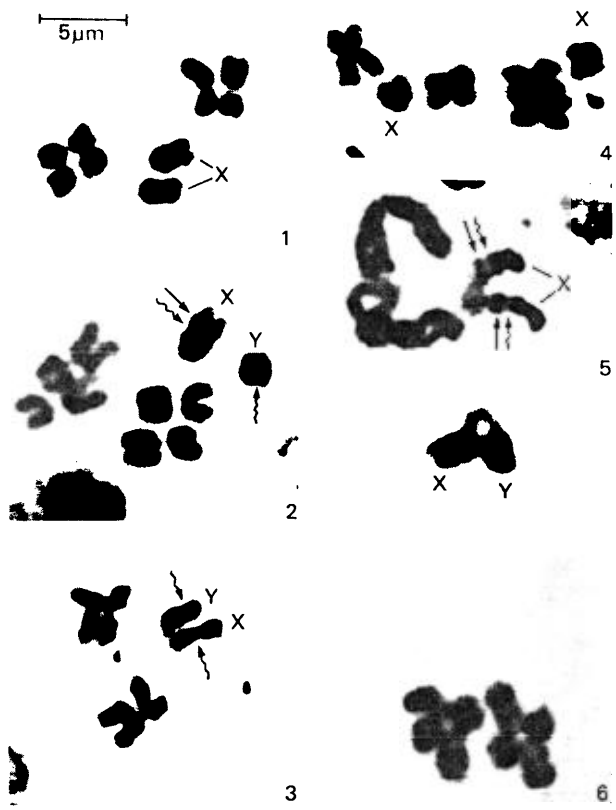
females from Nakhon Ratchasima Province (Wilkinson *et al.*, 1972); (2) *An. takasagoensis* (Taitung: Peiyuan Village strain), kindly supplied by Dr. J. C. Lien, Taipei, Taiwan; and (3) the Perlis form, initiated in 1966 from specimens collected near Padang Besar, Perlis State, Malaysia, which was kindly supplied by Mr. Cheong Weng Hooi, Institute for Medical Research, Kuala Lumpur, Malaysia. The first 2 colonies are maintained by artificial mating as described by Ow Yang et al. (1963), while the last is a natural mating colony.

Brain ganglion preparations for metaphase chromosomes were made from late 4th-stage larvae after pretreatment with a 0.1% colchicine solution. Orcein squash preparations were made according to a modified method described by Baimai (1975). This technique yields a large number of well-spread sister chromatids of prophase and metaphase chromosomes which frequently show distinct heterochromatic regions. The term heterochromatin used in this report refers to a region of a chromosome during prophase and early metaphase that remains condensed and stains darkly when orcein and Giemsa are used during the conventional preparation of mitotic chromosomes. Thus, a heterochromatic region of a chromosome is clearly visible at the prophase or early metaphase, but is less conspicuous later in metaphase when the whole chromosome is more uniformly condensed.

Preparations of meiotic chromosomes from pupal testes and salivary gland chromosomes from 4th-stage larvae were made using slight modifications of the standard orcein squash techniques (French *et al.*, 1962). All observations and photomicrographs were made from fresh temporary squash preparations using a green filter. Photomicrographs of metaphase and salivary chromosomes were recorded on Kodak high contrast copy and Panatomic film, respectively.



Figs. 1-6. Photomicrographs of metaphase or prometaphase chromosomes of *An. dirus* (1-3) and *An. takasagoensis* (4-6): (1) ♀ neuroblast; (2) ♂ neuroblast; (3) ♂ testis, metaphase II of meiosis; (4) ♀ neuroblast; (5) ♀ prometaphase neuroblast; (6) ♂ testis, diakinesis, meiosis I. Intercalary heterochromatin and secondary constrictions are indicated by arrows and wavy arrows, respectively.

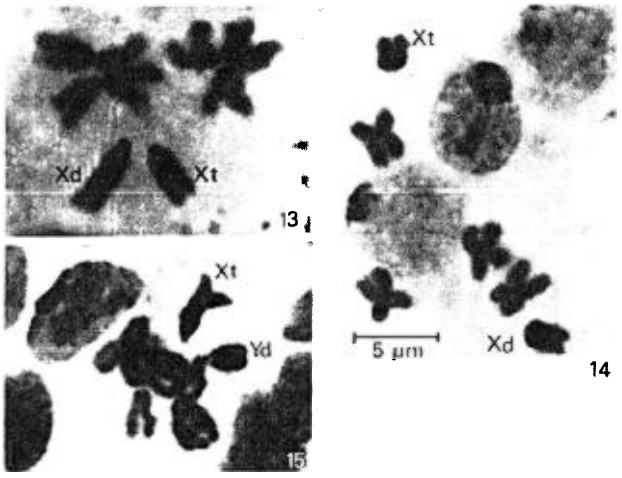
Results

The general metaphase karyotype of the three taxa is similar, consisting of two pairs of large submetacentric autosomes and one pair of telocentric or acrocentric rod-shaped sex chromosomes (Figs. 1-9). The term telocentric is used here for the rod-shaped sex chromosomes, because extremely short arms were not resolved under light microscope. These chromosomes, however, probably do not possess truly terminal centromeres. This interpretation is supported by meiotic chromosome preparations from pupal testes that always manifest a lightly staining bridge between the centromeric ends of the X and Y chromosomes (Fig. 6). Such an association cannot reflect the occurrence of a chiasma since it persists at second metaphase (Fig. 3) following an equational separation of the XY bivalent at first anaphase.

The distinct size and shape differences for the X and Y chromosomes of the three sibling species are described below.

Anopheles dirus shows telocentric X and Y

chromosomes. The latter is considerably shorter than the former and is, as a rule, entirely heterochromatic (Fig. 2). Although numerous pupal testes preparations were made for *An. dirus,* we were unable to find the small dot-like Y previously reported for this species (as *balabacensis)* by Aslamkhan & Baker (1969). The X chromosome is in turn approximately 0.80 the length of the shorter autosome (II). It is interesting to note that approximately 0.50 of the X chromosome toward the centromeric end is heterochromatic. This is clearly visible in early metaphase preparations (Fig. 10, 11). In addition, the X chromosome exhibits a conspicuous secondary constriction near the middle of the chromosome arm giving it the spurious

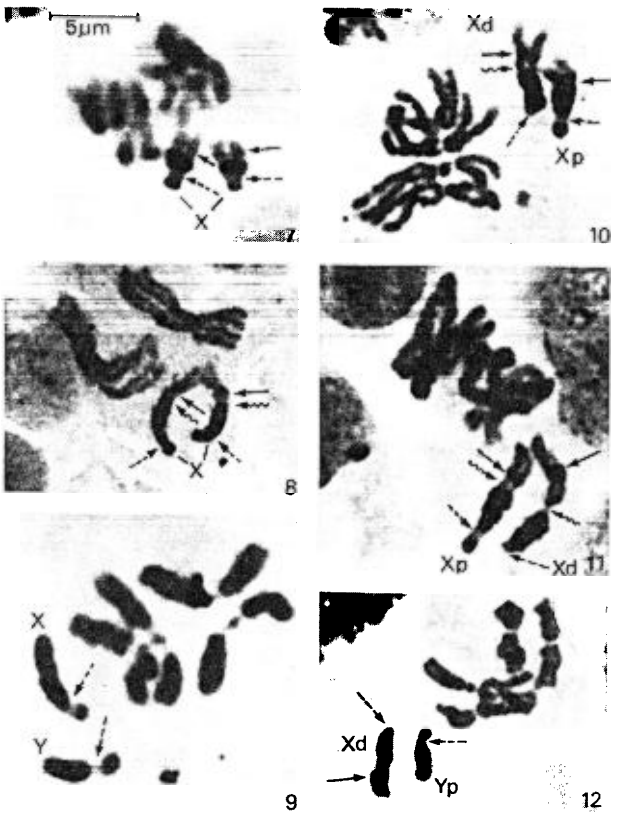


Figs. 13–15. Photomicrographs of neuroblast chromosomes of F_1 hybrids of ♀ *takasagoensis* × ♂ *dirus;* (13) F_1 ♀; (14) F_1 ♀; (15) F_1 ♂.

appearance of a metacentric in some preparations (Fig. 11). Furthermore, a small block of constitutive heterochromatin is intercalated in the euchromatic arm, and is readily seen in the less contracted preparations (cf. Fig. 2 with 10 and 11). The telocentric Y chromosome also exhibits a secondary constriction near the middle of the chromosome arm (Figs. 2, 15), but this is not as obvious as that found in the X chromosome.

The metaphase karyotype of *An. takasagoensis* (Figs. 4–6) is similar to that of *An. dirus.* The telocentric X and Y chromosomes are however, considerably shorter than those of *Am. dirus* and the Perlis form, although the submetacentric autosomes are similar in all 3 species. The X chromosome is approximately 0.65 the length of the shorter autosome and contains a considerable amount of centromeric heterochromatin (Fig. 5). The X chromosome of *takasagoensis* is clearly shorter than that of *dirus* as seen in a brain metaphase preparation of their F_1 hybrid (Figs. 13, 14). The X chromosome also exhibits a prominent secondary constriction and a small portion of intercalated heterochromatin in the euchromatic arm (Figs. 5, 15). The telocentric Y chromosome is clearly shorter than the X and is totally heterochromatic. A secondary constriction was frequently observed in the Y chromosome.

Surprisingly, the Perlis form exhibits a totally different type of X chromosome from the other 2



Figs. 7–12. Photomicrographs of metaphase and prometaphase of *An. balabacensis* (Perlis form) (7–9) and F_1 hybrids of ♀ *dirus* × ♂ *balabacensis* (Perlis form) (10–12): (7) ♀ neuroblast; (8) ♀ prometaphase neuroblast; (9) ♂ testis spermatogonial anaphase; (10, 11) F_1 ♀ prometaphase neuroblast; (12) F_1♂ neuroblast. Intercalary heterochromatin, centromeres and secondary constrictions are indicated by arrows, broken arrows, and wavy arrows, respectively.

species. It is a large acrocentric, or small submetacentric (Figs. 7, 9). The short arm is entirely heterochromatic, while at least 0.50 of the centric portion of the long arm is heterochromatic. The X chromosome of the Perlis form also exhibits a small amount of intercalated heterochromatin (Figs. 7, 8), as seen in the other two taxa. The centromeric constriction is more prominent than the secondary constriction and is usually observed in preparations. The X chromosome is approximately 0.80 the length of the shorter autosome, and is apparently the same size as the X of *dirus* (Figs. 10, 11). The Y chromosome is similar to the X in shape, but slightly shorter (Figs. 9, 12).

The metaphase chromosome configurations of these three closely related taxa are summarized in Figure 16.

If the polytene X chromosome is divided into 6 zones beginning at the distal end of the euchromatic section of the X (Baimai *et al.*, 1980) then all three taxa show similar banding patterns for zones 1–5. However, the taxa are strikingly different in zone 6 toward the centromeric end. Such differences are easily observed in F_1 hybrid X chromosomes (Figs. 17, 18). In general, zone 6 of *dirus* (Baimai *et al.*, 1980) is the longest, that of *takasagoensis* slightly shorter (Fig. 17), while that of the Perlis form is the shortest and only approximately 0.5 the length of that of *dirus* (Fig. 18). In addition to length, the banding sequences in zone 6 are apparently different in the 3 taxa, and they completely fail to synapse in F_1 hybrids.

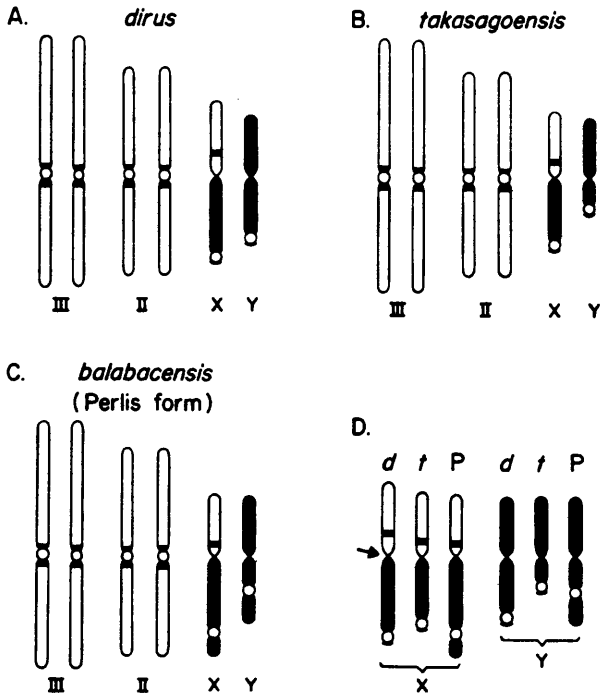
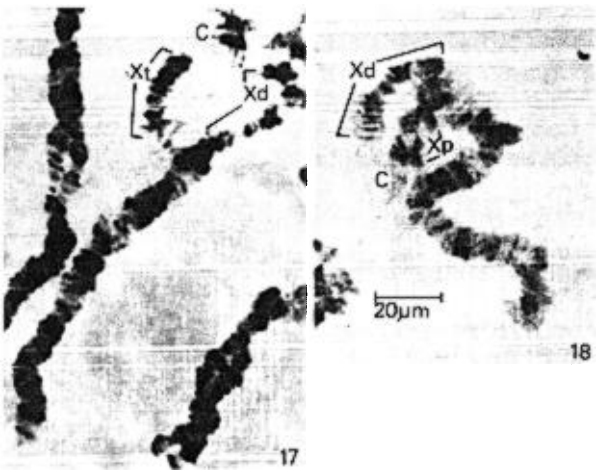


Fig. 16. Diagrams of metaphase karyotypes: (A) *An. dirus*, (B) *An. takasagoensis*, (C) *An. balabacensis* (Perlis form) and (D) sex chromosomes for the three species. Dark areas represent constitutive heterochromatin. An arrow indicates position of the secondary constrictions of the sex chromosomes of all 3 species.

Discussion

The cytological differentiation and heterochromatic composition of the X chromosomes observed in this study are of primary importance for understanding the chromosomal variation within the Balabacensis complex. Our studies of the salivary gland chromosomes strongly support these findings. Based on the standard salivary chromosome map of *An. dirus* (Baimai *et al.*, 1980), the X chromosome of all three species are very similar in zones 1–5 (Figs. 17, 18). The junction between zones 5 and 6 is apparently a very weak connection as zone 6 of the X breaks off in most preparations. This weak point of the X chromosome may cor-



Figs. 17–18. Photomicrographs of salivary gland chromosome X of the F_1 hybrids between ♀ *takasagoensis* × ♂ *dirus* (17) and ♀ *dirus* × ♂ *balabacensis* (Perlis form) (18). C indicates chromocenter.

respond with the secondary constriction observed in the metaphase chromosomes of the 3 species. On the other hand, it could coincide with the region of intercalated heterochromatin found in the euchromatic arm. This latter interpretation is more likely since heterochromatin does not undergo polytenization to the same extent as euchromatin in the salivary chromosomes (Lakhotia, 1974). Thus, the location of the heterochromatic portion could form a weak point in the salivary polytene chromosome.

The gain or loss of a small portion of chromosome is not necessarily a correlate of speciation (Swanson, 1965). However, the present data indicate that the evolution of the sex chromosomes of the species in the Balabacensis complex probably involves either the gain of heterochromatin or a pericentric inversion. If the ancestral sex chromosomes of these taxa were short and telocentric or somewhat similar to those of *takasagoensis,* the X and Y chromosomes of *dirus* possibly arose through the acquisition of extra heterochromatin. Likewise, the X chromosome of the Perlis form could have arisen by the acquisition of extra heterochromatin, which subsequently formed a short heterochromatic arm. However, the submetacentric Y chromosome of the Perlis form could have arisen from an ancestral chromosome either by the acquisition of extra heterochromatin, or by a pericentric inversion. Pericentric inversions were commonly induced in *An. culicifacies* Giles, by irradiation (Baker *et al.,* 1978), however the survival rate of such chromosomal aberrations was very low. On the other hand, the known natural occurrence of pericentric inversions in *Anopheles* is extremely rare (Coluzzi & Kitzmiller, 1975).

Cytological and cross-mating evidence (in preparation) suggests that *dirus* and *takasagoensis* are more genetically compatible with each other than either is with the Perlis form. Geographically, we consider *dirus* and the Perlis form to be sympatric in the vicinity of the Thai-Malaysian border, while *takasagoensis* is an insular species that is confined to the island of Taiwan, and apparently isolated from all other members of the Leucosphyrus group.

In summary, the present evidence supports the results of recent morphological studies (Peyton & Harrison, 1979, 1980), and cytogenetic and cross mating studies (in preparation) recognizing these three taxa as full-fledged species. However, although the Perlis form is distinguished by metaphase karyotype, morphological characters and a vigorous stenogamous mating behavior, it is currently known only from colonies. Accordingly, the Perlis form should not receive official taxonomic recognition until a naturally occurring wild population has been located and studied. Further extensive investigations have been initiated on the systematics of additional taxa in the Balabacensis complex, and to evaluate the role of each taxon of the complex in the transmission of human malaria parasites in Southeast Asia.

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